INITIATION OF DEVELOPMENT IN THE EGG OF *ARBACIA*.

II. Fertilization of Eggs in Various Stages of Artificially Induced Mitosis.

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A few summers ago the writer made certain experiments with various agents that caused the breakdown of the intact nucleus of the mature uninseminated eggs of *Arbacia* and of *Echinarachnius* with consequent liberation of the chromosomes in the cytoplasm. During the season of 1921 at the Marine Biological Laboratory, Woods Hole, Mass., opportunity presented itself to repeat some of these experiments as part of a detailed study of the effects of sea-water in varying degrees of hypertonicity on the uninseminated *Arbacia* egg. The present paper deals with the results obtained with one concentration only: sea-water made hypertonic by the addition of NaCl or KCl (in the proportion of 8 parts 2½ *M* salt to 50 parts sea-water). This report aims to set forth (1) that exposure to this hypertonic sea-water gives cleavage and a small per cent. of plutei, and (2) that eggs following treatment with this hypertonic sea-water on return to normal sea-water are capable of fertilization during any stage of the first cleavage mitosis except the telophase. It is this second finding which would seem to make this report of some interest: it suggests another method of attacking the problem of fertilization in the egg of *Arbacia*.

I.

The initiation of cell division in the egg of *Arbacia* by means of hypertonic sea-water is too well known to merit more than the briefest description. The pioneer work on this subject is, of course, that of Morgan; it was Loeb, however, who first produced larvæ with this method. Wilson has given an excellent account of the cytology of the egg of *Toxopneustes* induced to develop by

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hypertonic sea-water. The present study has nothing to add to these and other accounts of experimental parthenogenesis in echinid ova.

Briefly, I found that among a given lot of eggs in normal sea-water following exposure to hypertonic sea-water some would divide and form plutei that failed to swim at the surface, and that among those eggs that did not show cleavage were some that appeared unaffected by the exposure, while others formed monasters. Prolonged exposure was found to induce numerous cytasters. The egg nucleus, so far as I could determine, undergoes no increase in size while the eggs are in the hypertonic sea-water. I was likewise able to confirm Moore's finding, namely, that these eggs in sea-water after exposure to hypertonic sea-water give off fertilizin. These eggs never form membranes, as is well known.

II.

Moore has shown in an important contribution to the analysis of fertilization that the eggs of *Arbacia* inseminated immediately on return to sea-water after exposure to hypertonic sea-water (in the proportion of 8 parts 2½ *M* NaCl to 50 parts sea-water) develop for the most part in greater numbers than similarly exposed eggs without insemination. These inseminated eggs produce membranes comparable to those found on normally fertilized eggs. Cytological study revealed that sperm penetrate these eggs treated with hypertonic sea-water, but play no active role.

For the most part my findings agree with those of Moore. Since, however, the object of my study was somewhat different from his, my methods differed. This difference in method may well account for those of my results that are at variance with his.

In his experiments Moore gave the eggs graded treatment with hypertonic sea-water and inseminated them *immediately* on return to sea-water. In my experiments, since the primary object was to learn the fertilization capacity of the egg of *Arbacia* whose nucleus, having broken down, was thus in one or another stage of mitosis, I inseminated the eggs *at intervals* following their return to normal sea-water. It may be that the egg should lie for a time in normal sea-water, subsequent to exposure to hypertonic sea-water, before insemination in order to regain its equilibrium with the normal

medium. If this be true, it would perhaps account for the fact that I obtained much higher percentages of cleavage and of plutei from my inseminations than Moore from his.

Moore, as mentioned above, found scant evidence in his cytological study of any pronounced activity of the sperm. However, I am sure that in my material the sperm is quite active; it may form an aster, swell, and form chromosomes. (Cf. Herbst's studies.)

The method employed in these experiments is simple. Eggs from one *Arbacia* are collected as free from cœlomic fluid as possible, washed, allowed to settle, and exposed to hypertonic sea-water (in the proportion of 8 parts 2½ *M* NaCl or KCl to 50 parts sea-water). After varying lengths of time eggs are removed to normal sea-water. When they have reached the desired stage—monaster, metaphase, etc.—a portion is lightly inseminated with fresh, clean sperm suspension. The development of the inseminated eggs is compared with that of the uninseminated—per cent. of membranes, cleavage, and swimming larvæ. The following table presents the essential results of some of these experi-

TABLE I.

COMPARISON OF THE EFFECT OF EXPOSING EGGS OF *Arbacia* TO HYPERTONIC SEA-WATER (IN THE PROPORTION OF 8 PARTS 2½ *M* NaCl* TO 50 PARTS SEA-WATER) ALONE WITH THAT OF INSEMINATING EGGS IN WHICH NUCLEAR CHANGES HAVE BEEN INDUCED BY HYPERTONIC SEA-WATER.

No. of Experiment.	Time in Minutes of Exposure to Hypertonic Sea-water.	Effect of Exposure to Hypertonic Sea-water as Revealed by Per Cent. of Cleavage and of Larvæ.		Time in Minutes after Exposure to Hypertonic Sea-water when Inseminated.	Nuclear State of Eggs when Inseminated.	Effect of Insemination as Revealed by Per Cent. of Cleavage and or Top Swimming Larvæ.	
		Cleavage.	Larvæ.			Cleavage.	Larvæ (Estimated).
1	40	23	11	152	Swollen nucleus; monaster; spindle.	98	95
2	30	2	5	60	Swollen nucleus; monaster.	96	90
3	30	14	8	45	Monaster; spindle.	90	85
4	81	47	14	65	Spindle; monaster.	93	90
5	60	14	8	80	Spindles; many cytasters.	68	60
6	54	38	15	50	Metaphase; cytasters.	60	40
7	105	8	3	105	Anaphase; telophase.	38	25

* KCl gives similar results.

ments. It should be pointed out that the hypertonic sea-water used in these experiments never gave membranes. Insemination in normal sea-water of eggs that have had treatment with hypertonic sea-water may give 100 per cent. membranes.

We thus see that the egg of *Arbacia* that has such treatment with hypertonic sea-water that mitosis starts up is capable of responding to insemination during any phase of mitosis except the terminal. So far as I have been able to determine, insemination resulting in sperm penetration and perfect membrane separation is as easy to obtain in the anaphase as in the normal resting stage. The plutei from these eggs are as viable as normal plutei.

With the onset of the cortical changes preceding first cleavage, these eggs fail to respond to insemination. So far I have not studied the effect of inseminating these eggs after first cleavage. According to Loeb, however, the blastomeres of the egg of the California sea-urchin induced to cleave through exposure to hypertonic sea-water separate membranes on insemination. Moore, on the other hand, was unable to obtain membranes after inseminating isolated blastomeres of *Arbacia* eggs induced to cleave by exposure to hypertonic sea-water.

I have in my work encountered eggs that simulate first cleavage; in such eggs one of the "pseudo-blastomeres" on insemination will form a membrane, cleave, and swim. The other member of the pair never shows any trace of development. Such eggs seemingly composed of two blastomeres may be easily produced in large numbers.

If eggs that have had an exposure to hypertonic sea-water be shaken gently or squirted through a pipette into normal sea-water, they appear as eggs in the two-cell stage. These pseudo-blastomeres may be equal in size or of all degrees of inequality in size.

Such eggs on insemination form membranes around one component only, regardless of its size. Thus one may get a large or small cleaving egg within a membrane and subsequently a swimming form, attached to an inert mass of cytoplasm without the vestige of membrane. The explanation of this condition is simple.

The hypertonic sea-water so alters the cortex of the egg that it breaks when the egg is shaken and allows an outflow of endoplasm. It is this endoplasm free of cortical material that fails to respond

to insemination. The egg nucleus may be located in either of the "pseudo-blastomeres"; or, as is frequently the case, it may be seen lying in the constriction between the "pseudo-blastomeres."

An interesting figure found in sectioned material deserves passing notice. In this case the spindle is in late anaphase; one pole with a chromosome group is in the larger of the "pseudo-blastomeres," which doubtless formed a membrane, since all the eggs in this lot had membranes, and the other pole with a group of chromosomes is in a minute protuberance that could easily pass as a polar body. A spermatozoön was found in the cortex at the pole opposite the "polar body." This picture may be worthy of more than passing comment.

Observations on various ova (*Myzostoma, Chætopterus, Dentalium, Amphioxus, Clepsine,* etc.) show that the ectoplasmic layer is absent at the outer end of the maturation spindle. Moreover, Chambers by microdissection has shown that the region over the outer end of the maturation spindle in the egg of *Cerebratulus* is very fluid. If we assume an absence of cortex in this region (or perhaps physical or chemical difference), polar-body formation would be comparable to this extrusion of endoplasm through the cortex that I have mentioned above.

Observations likewise show that in various ova (those of *Ciona, Cynthia, Chætopterus, Nematodes,* etc.) the cortex as well as the endoplasm flows toward the vegetative pole. In *Chætopterus,* for example, the ectoplasmic waves are clearly visible in the living egg. It is not wholly impossible, therefore, that the definitive location of the maturation spindle at the animal pole is owing as much to the energy of these downward movements as to the movement of the spindle itself. It is as if the egg substance *flows away* from the animal pole, leaving the spindle behind. On this assumption the size of the polar body would thus depend upon the size of the more fluid ectoplasmic defect at the animal pole, the energy of the downward movement of the ectoplasm, or both. Conklin's production of large polar bodies in the egg of *Crepidula* through centrifuging might be cited as evidence that an unusual bulk of material may be thrust out of the egg as a polar body.

Usually polar bodies do not fertilize. In ova in which maturation normally takes place before fertilization this failure to fer-

tilize may be due to absence of fertilizin. If normally fertilization occurs during first maturation, the failure of polar bodies to fertilize may be due to the fixation of fertilizin; polar bodies are thus sterile as are all parts of the egg. If it prove that polar bodies lack cortex and are really endoplasmic, this would be suggestive as to the location of fertilizin in such ova, as, for example, *Asterias,* in which fertilization may take place at various stages of maturation.

III.

The experiments above noted show that eggs of *Arbacia* induced through treatment with hypertonic sea-water to initiate mitosis after return to sea-water are capable of giving a response to insemination similar to that in normal fertilization. This gives rise to several considerations. We may discuss these in turn.

1. In the first place, the stage in mitosis through which an egg is passing at the time of insemination is of no consequence for complete cortical response. Eggs in any stage of mitosis (except the telophase) respond completely to insemination whether or not the spermatozoa entering such eggs take part in the ensuing divisions. This would indicate that physical or chemical changes set up in the cytoplasm during mitosis constitute no bar to fertilization. In this respect changes set up by the first cleavage mitosis do not differ from those in the maturation mitoses in those eggs that normally take in sperm before complete maturation. Thus changes in permeability, rate of oxidation—themselves held as "causes" of fertilization—do not interfere with the cortical reaction to sperm.

If we define fertilization, in terms of this cortical phenomenon, as an instantaneous reaction between some ovogenous substance and the spermatozoön at the time of insemination, the experiments here reported are again suggestive. Of course, the reader may not accept this definition of fertilization; indeed, it may turn out to be wholly fallacious. Let us, however, for the sake of argument, assume that the definition is correct; that the primary aim in the fertilization process is the incorporation of the sperm head as part of the zygote nucleus, thus insuring equivalence of maternal and paternal chromatin in heredity; and that the reaction at the egg

cortex between fertilizin and sperm guarantees this incorporation. Cell division is thus the end result of fertilization. On this hypothesis, then, the experiments here reported suggest a mode of attacking the fertilization-reaction apart from cell division.

In the third place, our results suggest the possibility of testing the validity of various theories of fertilization: for example, the oxidation (Loeb), the permeability (R. S. Lillie, McClendon, Gray), and the viscosity (Fischer and Ostwald, Heilbrunn) theories. If, after treating *Arbacia* eggs with hypertonic sea-water of the strength used in the experiments cited above, we were to find *no* increase in permeability or oxidation, for example, but on inseminating these eggs were to find *pronounced* increase, the case for such increase as the "cause" of fertilization would demand a hearing. But hypertonic sea-water alone of the strength used in our experiments increases the rate of oxidation and permeability. Moreover, this being true, even if there were an additional increase in the rate of oxidation (or permeability) following the insemination of these eggs previously exposed to hypertonic sea-water, and if this increase plus that due to hypertonic sea-water alone were equal to the increase of an equal number of eggs from the same female following normal insemination, we could not hold the oxidation (or permeability) theory as proved. For the fact still remains that oxidation (or permeability) increase is not inseparably bound up with the fertilization-reaction.

The case is similar with the viscosity theory of fertilization. Mrs. Andrews long ago showed by means of subjecting eggs to pressure that a rhythm of viscosity changes accompanies the cleavage process. It may also well be that following the liquefaction of the cortex, as in the egg of *Echinarachnius,* the ectoplasm gels. Also, Lillie finds that in unfertilized eggs of *Chætopterus* in the mesophase of first maturation, though stratification of the endoplasm readily results from centrifuging, the ectoplasmic layer remains unaffected. That fluid substance diffuses from nucleus into cytoplasm is of course well known. This has been shown especially for the egg in the germinal vesicle stage. Says F. R. Lillie on this point: "During this period of diffusion of the fluid substance of the germinal vesicle and the ensuing polarization of the ectoplasm and endoplasm, the protoplasm as a whole possesses a

much higher degree of fluidity than before" (Lillie, '06, page 176). "The strongest evidence for greater fluidity at this time is found in the fact that the ectoplasmic spherules are much more numerous and smaller than they were previously or than they are subsequently. Evidently there is a reversible process of coagulation concerned, the spherules breaking down into smaller particles as the fluidity increases and setting or coagulating again by a process of fusion."

"If eggs are allowed to stand eight to fifteen minutes in sea-water after being taken from the female so that in some the germinal vesicle is intact and in others broken down, the latter always show stratification more or less pronounced after centrifuging and the former never show it. . . . It would seem that the endoplasm has become less viscid as a result of the diffusion of substance from the germinal vesicle so as to permit of a closer aggregation of the yellow granules."

Let us, however, waiving this demonstration of a difference in the physical make-up of the cortex and endoplasm of the *unfertilized Chætopterus* egg, as well as Chambers's observation that the gelation succeeding insemination is localized in the region of the sperm aster, assume that in the *Arbacia* egg a gelation follows the liquefaction of the cortex. But the viscosity theory of fertilization is not thus made more tenable.

According to the viscosity theory (Heilbrunn), initiation of development by artificial agents or by sperm involves coagulation of the eggs; the mitotic spindle probably arises as a direct result of this coagulative change. But in the experiments cited above in this paper eggs that have first cleavage spindles (cf. many eggs normally fertilized during maturation phases) respond to insemination. Such eggs will develop without the cortical changes that in normal eggs follow insemination. We should, however, point out that, according to Heilbrunn, hypertonic sea-water coagulates the egg.[1] It may thus be argued that those eggs with spindles are only incompletely activated if they give cortical response to insemination. The truth, though, is quite otherwise. The cortical

[1] All that Heilbrunn shows is that the eggs are more viscous while *in hypertonic solutions*. We are told nothing of their history after return to normal sea-water.

changes induced by sperm are not merely quantitatively but qualitatively different from those induced by hypertonic sea-water; the cortex is liquified following insemination with resulting membrane separation, but it is thickened by hypertonic sea-water. If anything were wanting to substantiate the view here presented, Heilbrunn's own findings would do so: many diverse agents besides hypertonic sea-water produce coagulation in the egg. That Heilbrunn does not show that all of these so-called agents of artificial parthenogenesis do actually produce cell division—and this according to his own definition of artificial parthenogenesis they should do—alone is fatal for the whole theory. In addition, it is not beyond the realm of possibility that many of these agents (distilled water acting upward to *four minutes,* toluene acting for *five minutes,* saponin acting for *five minutes,* etc.) simply induce death changes—of no significance whatsoever for the problem of fertilization.

There remains the fertilizin theory (Lillie, '14). Here we find no difficulty. Eggs of *Arbacia* following exposure to hypertonic sea-water possess fertilizin despite mitotic changes. Such eggs on insemination, as we should expect, give complete cortical reaction. Without insemination such eggs develop. *Fertilizin fertilizes the sperm* so that it may by reacting with aster-forming substance of the egg start up division in which the egg nucleus takes part. But eggs that are already induced to develop by hypertonic sea-water do so through localization of aster-forming substance around their nuclei. Sperm entering such eggs may still react with fertilizin, thereby setting free cortical changes leading to membrane separation.

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